Journal of Medical Virology 55:42–50 (1998)

G_1 Phase Dependent Nuclear Localization of Relaxed-Circular Hepatitis B Virus DNA and Aphidicolin-Induced Accumulation of Covalently Closed Circular DNA

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During chronic hepatitis B virus (HBV) infection, virus persistence relies on the maintenance of a pool of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes. To achieve this, HBV DNA has to be transported from the cytoplasm to the nucleus. By carrying out subcellular fractionation experiment, both of the relaxed-circular (RC) and single-stranded (SS) HBV DNA were found in the cytoplasm whereas only RC form could be detected in the nucleus of a hepatoblastoma cell line (HepG2) stably producing HBV. This fraction of nuclear RC viral DNA was clearly demonstrated in the G_1 but not S phase of synchronized HepG2 cells. Conversely, the relative amount of cytoplasmic RC viral DNA in the S phase was larger than that in the G1 phase. Although no cccDNA could be detected in HepG2 cells without synchronization, an increasing amount of cccDNA in the nucleus was demonstrated after prolonged incubation of the cells in aphidicolin. Finally, by undertaking in situ hybridization using a probe specific to plus-strand HBV DNA, nuclear viral DNA was detected predominantly in the G1 phase of HepG2 cells. In summary, the results indicated that only RC but not SS form of HBV DNA was localized to the nuclei of HepG2 cells. The nuclear localization occurred preferentially in the G_1 but not S phase and prolonged treatment with aphidicolin resulted in accumulation of nuclear cccDNA. ***J. Med. Virol. 55:42–50, 1998.***

KEY WORDS: nucleus; cell cycle; viral DNA

INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic hepatitis. Sequelae such as liver cirrhosis and hepatocellular carcinoma occur in the late stage of the disease [Chen, 1993; Yoffe and Noonan, 1993]. The DNA of HBV is a small, partially double-stranded molecule approximately 3.2 kb in length which is held in a circular conformation by a short cohesive overlap between the 5′ ends of the two DNA strands [Ganem and Varmus, 1987]. In persistently infected cells, virion DNA synthesis occurs in the cytoplasm [Blum et al., 1984; Summers and Mason, 1982]. Using pregenomic RNA as a template and viral polymerase as a reverse transcriptase and protein primer, a single-stranded (SS) viral DNA is first synthesized in viral nucleocapsid particles [Wang and Seeger, 1992; Zoulim and Seeger, 1994]. The synthesis of minus-strand DNA which serves as a template for plus-strand DNA synthesis is important for the maturation of nucleocapsid particles [Gerelsaikhan et al.,1996]. After completion of the plus-strand DNA synthesis, a relaxed-circular (RC) virion DNA results, which still contains the covalently bound protein primer at the 5′ end of the minus strand [Summers, 1975; Gerlich and Robinson, 1980; Molnar-Kimber et al., 1983; Wang and Seeger, 1992]. The nucleocapsids containing plus-strand viral DNA can be packaged and exported as mature virions. Alternatively, the viral DNA can be transported into the nucleus to form covalently closed circular (ccc) HBV DNA, which is presumably the template for transcription of viral pregenomic RNA [Mason et al., 1983; Tuttleman et al., 1986]. The size of this pool of cccDNA in the nucleus is not controlled by semiconservative replication, but by nuclear transport of cytoplasmic viral DNA [Tuttleman et al., 1986; Wu et al., 1990]. Therefore, it is important to identify cellular and/or viral factors that determine whether the nucleocapsids in the cytoplasm are matured to exit the cells or are recycled to the nucleus to maintain the pool of cccDNA.

Contract grant sponsor: National Science Council Republic of China, Taiwan; Contract grant numbers: NSC84-2331-B182-042-MH; NSC86-2315-B-182A-001-MH.

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Accepted 6 January 1998

In duck hepatitis B virus, large envelope protein was shown to regulate cccDNA amplification [Summers et al., 1990; Summers et al., 1991; Lenhoff and Summers, 1994]. Similarly, human HBV large envelope protein has also been shown to retain the core particles in the cytoplasm [Yeh et al., 1994]. Obviously, this protein plays an important role in inhibiting the amplification of cccDNA. Core protein itself has also been suggested to play a role in regulating cccDNA levels [Eckhardt et al., 1991; Yeh et al., 1993; Kann and Gerlich, 1994; Liao and Ou, 1995]. To carry the viral DNA through the nuclear pore, the nucleocapsids have to be at least partially dissembled into sub-particles, since an intact core particle cannot enter the nuclei in the transgenic mice experiment [Bock et al., 1994; Guidotti et al., 1994]. Alternatively, the nucleocapsids may dissemble to release the viral DNA, which is sufficient for the nuclear entry of HBV DNA, while nuclear transport of the remaining core protein is a by-standing event [Birnbaum and Nassal, 1990; Yu and Summers, 1991; Guidotti et al., 1994; Kann et al., 1997].

Although different mechanisms may be involved in different systems, the nuclear entry of HBV core protein is believed to be cell cycle related [Yeh et al., 1993; Guidotti et al., 1994; Chu et al., 1995]. Since core protein might play a crucial role in cccDNA amplification, it is important to understand whether accumulation of cccDNA in the nucleus is also cell cycle related. Recently, it was shown that a cell cycle blocker, *n*-Butyrate, inhibits early amplification of duck hepatitis B virus cccDNA [Turin et al., 1996]. Although this information is important, the experiment was carried out using duck hepatocytes, in which core protein does not normally enter nuclei. We investigated the subcellular transport of HBV DNA in HepG2 cells during different phases of cell cycle.

MATERIALS AND METHODS

Cell Lines and Cell Cycle Synchronization

HepG2 cells were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum. The HepG2.HBV stable transformant was established by transfecting HepG2 cells with pSV2a-neo-HBV2 (ayw subtype, GenBank accession number U95551, a gift from J. Ou, USC). This plasmid contained a HBV head to tail dimer connected at *Eco*R I sites and a neomycin resistent gene driven by SV40 promoter. After G481 selection, the clone secreting highest amount of HBsAg was expanded for our experiments. Cell cycle synchronization was performed according to the procedure described previously [Yeh et al., 1993]. Briefly, cells were incubated in DME medium without serum for 30 hr. This resulted in synchronization of cells in G_o phase of the cell cycle. The cells were then initiated with DME medium containing 25% (v/v) fetal bovine serum (FBS). For synchronization with aphidicolin, cells were incubated with DME medium containing 10% fetal bovine serum and 2 μg of aphidicolin per ml for 48 hr. This resulted in synchronization of cells in the late G_1 phase. The cells were either analyzed at this time point, allowed to enter the S phase by further incubation in the same medium without aphidicolin, or further incubation in the medium containing aphidicolin for up to 9 days.

Subcellular Fractionation

For subcellular fractionation, cells were rinsed with phosphate-buffered saline (PBS) twice and lysed with 0.5 ml of TBS (10 mM Tris-HCl [pH 7.2], 150 mM NaCl) containing 0.5% Nonidet P-40 (NP-40) [Yeh et al., 1990]. The nuclear fraction (NP-40-insoluble fraction) and the cytoplasmic fraction (NP-40-soluble fraction) of the cell lysates were separated by a brief centrifugation in a microcentrifuge at 1,500 × *g*. The nuclear fraction was washed twice with 0.5 ml of the same buffer and the supernatant of the second wash (W2) was collected. The post-washed nuclear fraction was solubilized in 1 ml of RIPA solution (10 mM Tris-HCl [pH 7.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). The cytoplasmic fraction was also mixed with an equal volume of RIPA buffer. To determine the concentrations of core protein, 200 μl of each fraction were used for RIA (Abbott HBe-RIA kit). To extract HBV DNA, an equal volume of lysis buffer (50 mM Tris-HCl [pH 7.2], 1 mM EDTA, 1% SDS, 0.45% NP-40, and 200 μg/ml protease K) was added and the mixture was incubated for 2 hr at 55°C. After extracted twice with equal volumes of phenol-chloroform and once with chloroform, the DNA was precipitated with acid ethanol and dissolved in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

[^{3}H]Thymidine Incorporation Assay

The [^{3}H]thymidine incorporation assay was carried out according to a procedure described previously [Yeh et al., 1993]. Briefly, the cells were labeled with [^{3}H]thymidine at a concentration of 25 μCi/ml (1 μCi = 37 kBq) for 30 min at selected time points after synchronization. The cells were then rinsed with PBS twice and lysed with 1 ml of 0.1 M NaOH containing 10 mM EDTA and 0.5% SDS. After heated at 70°C for 30 min, the cell lysate was mixed with 100 μl of 100% CCl_3COOH and incubated at 4°C overnight. The precipitates were then collected on GF/C filter discs (Millipore), rinsed with 5% CCl_3COOH solution and then with 95% ethanol, air-dried, and scintillation-counted.

Flow Cytometric Analysis

Synchronized cells were detached from the plate by trypsinization, washed in ice-cold PBS, and fixed by 100% ethanol. After staining with propidium iodide, cells were analyzed using a Becton Dickinson FACSort flow cytometer [Freytag SO, 1988]. To calculate the percentages of cells in different cell cycle phases, cells without synchronization was first analyzed by flow cytometry to identify the peaks representing the G_1 and G_2 phases. The readings of DNA content for the G_1 and G_2 phases were assigned as 1 and 2 copies/cell. Cells with DNA content between 0.75–1.25, 1.25–1.75, and

1.75–2.25 copies/cell were calculated as G_1, S and G_2 phase cells.

Detection of HBV DNA with Digoxigenin-Labeled Probe

To detect HBV DNA, the extracted samples were loaded onto a 1% agarose gel for electrophoresis before blotted onto a nitrocellulose membrane. HBV DNA was then detected by hybridization with a digoxigenin-labeled probe. To prepare the digoxigenin-labeled probe, polymerase chain reaction (PCR) was performed using a plasmid, pECE-C, as the template [Yeh et al., 1990]. This plasmid contained a complete HBV genome [Valenzuela, et al., 1980]. The primers used were 5′-CATTGCTCACCTCACCATAC-3′ (sense; nt. 2042 to 2061) and 5′-GAAGGAGTTTGCCATTCAGG-3′ (anti-sense; nt. 2548 to 2529). To obtain a more specific probe, TaKaRa Ex Tag polymerase (Takara Shuzo Co., Shiga, Japan), which was capable of proof-reading, was used in this experiment. PCR reaction was performed as previously described except that 1/3 of dTTP in the dNTP mixture was replaced by Digoxigenin-11-dUTP (Boehringer Mannheim GmbH, Germany) [Yeh et al., 1994; Yeh et al., 1997]. After hybridization, the signal was detected by DIG Luminescent Detection Kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instruction.

Specific Detection of Plus-Strand HBV DNA

To generate a digoxigenin-labeled, single-stranded probe which hybridized specifically to plus-strand HBV DNA, a DNA fragment containing HBV core coding region was first obtained by PCR (Fig. 4). The primers used for this PCR were primer a, 5′-AGGCTGTAGGCACAAATTGG-3′ (sense, nt. 1781–1800), and primer b, 5′-GAAGGAGTTTGCCATTCAGG-3′ (anti-sense, nt. 2548–2529). The plasmid, pECE-C, is used as template. After gel-purification, this DNA fragment was further used as a template for single primer PCR. The primer used was primer Pr, 5′-GTATGGTGAGGTGAGCAATG-3′ (anti-sense, nt. 2061–2042). The dNTP mixture used containing Digoxigenin-11-dUTP as described in the previous section. Two single-stranded HBV DNA were also generated by single primer PCR as controls: primer p, 5′-GCCTCCAAGCTGGCCTTGG-3′ (sense, nt. 1871–1890), and primer m, 5′-ACCACAATAGTTGCCTGATC-3′ (anti-sense, nt. 2210–2191) were used to generate plus and minus strand DNA controls, respectively.

In Situ Hybridization

The digoxigenin-labeled, plus-strand specific probe described above was used further for in situ hybridization. Cells grown on cover slips were fixed in acetone at –20°C for 1 min. The slide was incubated carefully in 0.2 M HCl at room temperature for 20 min without shaking, rinsed with pure water, and then incubated in 2 × SSC at 60°C for 30 min. After pretreatment with 1 μg/ml of protease K and 5 U/ml of RNAse A in PBS at 37°C for 15 min, the slide was dehydrated in graded series of ethanol and air dried. The slide was then incubated in prehybridization solution (0.2% SDS, 10 × Denhardt's solution, 6 × SSC) at 65°C for 1 hr before hybridized in hybridization solution (10× Denhardt's solution, 6 × SSC) containing the gel-purified, digoxigenin-labeled probe (1 μg/ml) at 40°C for 18 hr. After hybridization, the slide was washed subsequently in 4 × SSC, 2 × SSC, and 0.2 × SSC for 5 min (2 times each) before incubation in modified TBS/Triton (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM MgCl2, 0.1% bovine serum albumin, 0.1% Triton X-100) at room temperature for 15 min. The slide was then incubated with Sheep anti-digoxigenin/AP conjugate and developed with Revealing reagent (In Situ Hybridization Detection Kit, British Biotechnology Products Ltd., UK).

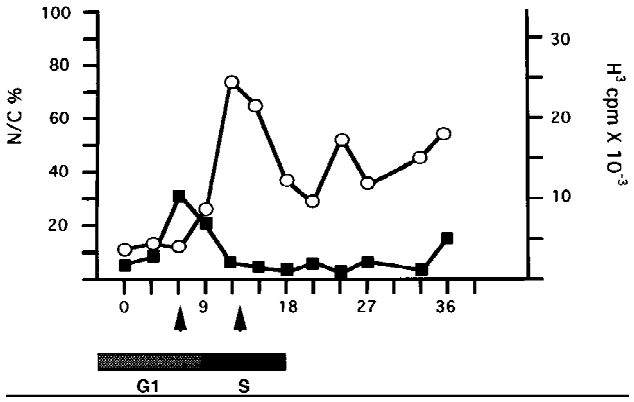

Fig. 1. Synchronization of HepG2.HBV cells by serum starvation. Cells were synchronized by serum starvation as described in Materials and Methods. Solid square: relative ratios of the amount of the core protein in the nucleus and that in the cytoplasm (N/C) determined by RIA. Empty circle: results of [^{3}H]thymidine incorporation assay. All data were means of three independent experiments. Horizontal scale: hours after supplement of 25% FBS. Periods of time in which cells were predominantly in the G_1 (shaded bar) or S phase (solid bar) determined by flow cytometry were marked. Arrowheads: 6 and 13 hr after supplement of serum.

Detection of cccDNA

To isolate cccDNA by Hirt fractionation, cells were lysed in a solution of 1% SDS, 1 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 5 mM EGTA. After incubation overnight at 4°C, the cellular DNA was pelleted at 10,000 rpm for 40 min at 4°C in a Sorvall HB-4 rotor. The Hirt supernatant was extracted twice with phenol, twice with phenol and chloroform and once with chloroform without previous protease K digestion. Nucleic acids were precipitated with acid ethanol and dissolved in TE for further analysis. To construct a control plasmid, pECE-fC, the *Bgl*II-*Bgl*II fragment (0.42 kb) from HBV core coding region was isolated and inserted into pECE-1. This plasmid was used to test the efficiency of cccDNA extraction.

RESULTS

Synchronization of HepG2.HBV Cells by Serum Starvation or Aphidicolin

To study the influence of cell cycle on the subcellular traffic of HBV DNA, a HepG2 cell line, HepG2.HBV,

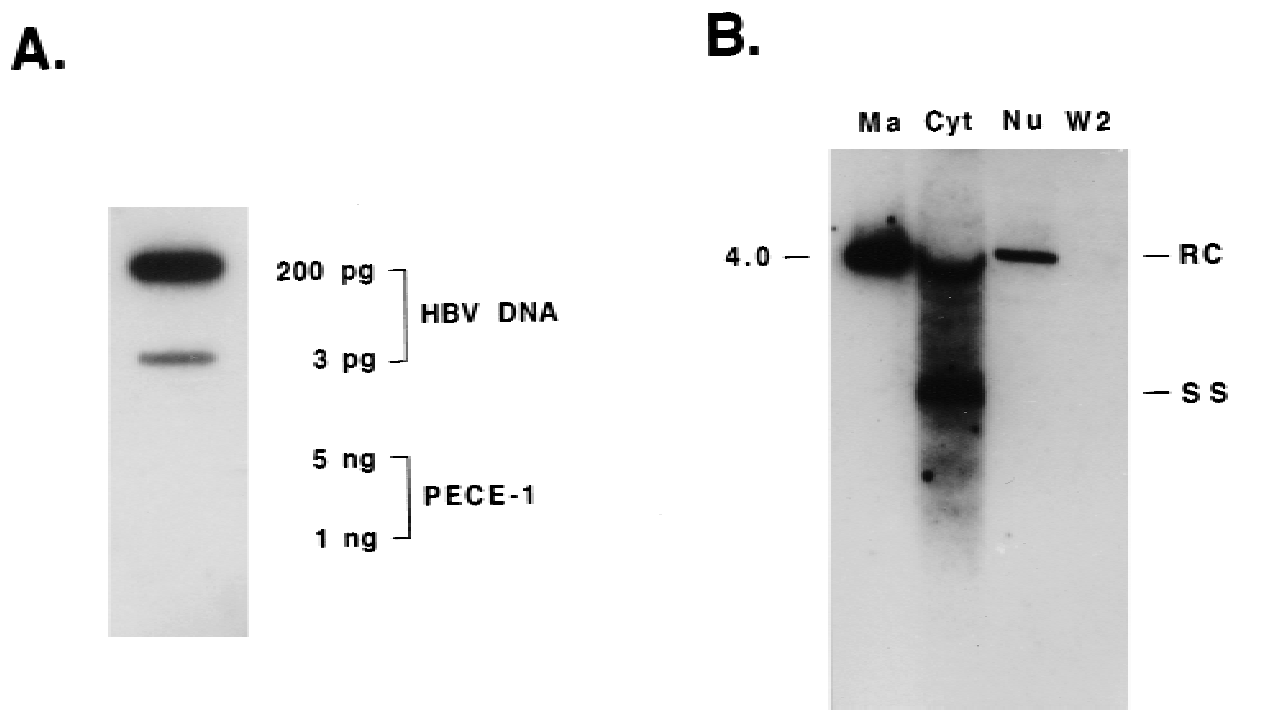

Fig. 2. Subcellular distribution of HBV DNA in HepG2.HBV cells. (A) Sensitivity of the digoxigenin-labeled probe. HBV DNA fragment isolated by enzyme digestion from pECE-C (Yeh et al., 1990), and a plasmid, pECE-1, served as the positive and negative controls, respectively. (B) Subcellular distribution of HBV DNA. Ma: marker, a *Ssp*I-*Xba*I (4.0 kb) fragment isolated from pECE-C; Cyt: cytoplasmic fraction; Nu: nuclear fraction; W2: the second wash solution; RC: relaxed-circular HBV DNA; SS: single-stranded HBV DNA.

capable of supporting HBV replication was established. As a first step, two different methods were used to synchronize the cells. Serum starvation for 30 hr resulted in a very low thymidine uptake, indicating that the cells entered the $G_{0/1}$ phase of the cell cycle. After supplement of 25% FBS, thymidine uptake started to increase at 9 hr and reach the peak at 12–14 hr (Fig. 1). Afterwards, the level of uptake never returned to the basal level, indicating poorly synchronization after the first S phase. Flow cytometer analysis of cellular DNA content revealed that at 6 hr, 89–95% and 5% of cells were in the G_1 and S phases respectively, whereas, at 13 hr, 20–35% and 40–55% (varied between different experiments) of cells were in the G_1 and S phase respectively. Synchronization by aphidicolin gave a similar result, with around 30% of cells remaining in the G_1 phase at 13 hr after removal of aphidicolin. Measurement of HBV core/e antigen by radioimmunoassay allowed us to calculate the ratio of core protein in the nucleus (N/C) over different time points. A small peak of nuclear core protein was only observed at 6 hr after serum supplement (N/C = 28–31%; Fig. 1).

Subcellular Distribution of HBV DNA

By carrying out subcellular fractionation followed by southern blot analysis, subcellular distribution of HBV DNA in HepG2.HBV cells was investigated. To ensure the nuclear fraction was not contaminated by the cytoplasmic fraction, the nuclear pellet was washed twice and the supernatant from the second wash was collected and analyzed in parallel. It was found that no HBV DNA could be detected in this wash solution (Fig. 2B). Two major HBV DNA species, migrating at the positions of about 3.8–3.9 and 1.6–1.7 kb were found in the cytoplasmic lane, corresponding to RC and SS forms of HBV DNA. A background smear and several poorly defined bands were also found, representing various forms of replication intermediates. The distortion of the band at 3.8–3.9 kb (RC) was likely caused by overloading of the sample, since loading of a smaller amount of sample (one fifth) resulted in non-distorted band (data not shown). Interestingly, on the nuclear lane, there is only one clear 3.9–4.0 kb band, which is likely the RC viral DNA. No other forms of HBV DNA can be detected. The specificity of the digoxigenin-labeled probe was illustrated in Fig. 2A (also in Fig. 3, control lanes).

Nuclear Localization of RC Viral DNA is Cell Cycle Dependent

After synchronization by serum starvation, HepG2.HBV cells at 6 (G_1 phase) and 13 (S phase) h after serum supplement were harvested for HBV DNA analysis (Fig. 3). Interestingly, the density of 3.8–3.9 kb species (RC) in the cytoplasm increased substantially when the cells progressed from the G_1 to S phase (3.0 to 4.2 folds of increase by densitometry), whereas the density of 1.6–1.7 kb species (SS) remained unchanged. Conversely, the 3.9–4.0 kb band (RC) in the nucleus was only detected in the G_1 phase but com-

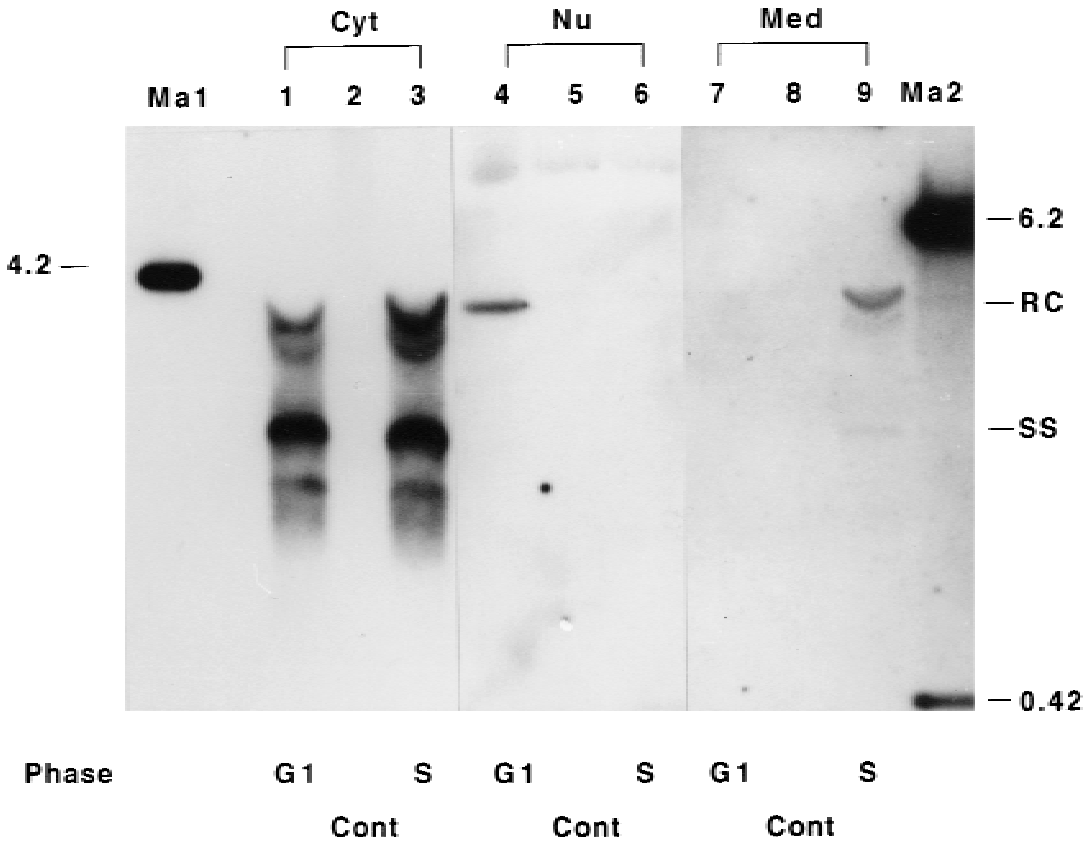

Fig. 3. Subcellular distribution of HBV DNA in HepG2.HBV cells during the G_1 and S phases. The *Bam*HI-SspI (4.2 kb), and *Bgl*II-*Bgl*II (6.2 and 0.42 kb) fragments isolated from pECE-C were used as markers (Ma1 and Ma2, respectively); Cyt: cytoplasmic fractions; Nu: nuclear fractions; Med: medium; HepG2.HBV cells in the 6 (G_1) and 13 (S) hr after serum supplement were used; the original HepG2 cells were used as controls (Cont).

pletely absent in the S phase. To determine the amount of HBV DNA secreted out of the cells during the G_1 and S phases, culture medium collected at 6 and 13 hr after serum supplement were analyzed. If the secretion rates were the same during these two phases, a two-folds increase of HBV DNA was expected. However, in repeated experiments, HBV DNA in the medium from the G_1 phase (6 hr) cells was in a very small amount if detected. Densitometry revealed an 18–55 folds of increase when the cells progressed from the G_1 to S phase. During serum starvation, no obvious cell lysis was visulized. However, to understand whether the increase of HBV DNA in the medium was due to core particles released from undetected dead cells, HepG2.HBV cells were also serum-starved for 36 and 43 hr and the medium was analyzed. It was found that HBV DNA in the medium was undetectable by Southern blot at these two time-points.

Detection of cccDNA in HepG2.HBV Cells after Prolonged Incubation with Aphidicolin

In the beginning of this study, every effort was made to detect cccDNA in HepG2.HBV cells but these failed. Since nuclear transport of RC viral DNA only occurred in the G_1 phase, a rapidly growing cell culture possibly did not have a G_1 phase long enough for RC viral DNA to be repaired and become cccDNA. We therefore tried to block the cells in G_1 phase for a longer period of time. In our initial experiments, it was found that HepG2.HBV cells started to die after 48 hr of serum deprivation, whereas in 10% of serum, aphidicolin was capable of blocking the cells for up to 6–8 days. When these cells were examined, cccDNA was detected at the third day and the amount continued to increase at the following four to eight day (Fig. 4A). Afterward, the amount decreased rapidly (cells died). The cccDNA from HepG2.HBV cells 4 days after aphidicolin treatment were also analyzed by *EcoR*I enzyme digestion (Fig. 4B). A band shift from 2 kb to 3.2 kb was observed, which confirmed the nature of this band (cccDNA).

Specific Detection of Plus-Strand HBV DNA and In Situ Hybridization Analysis

To solidify the observation that the entry of RC viral DNA was cell cycle dependent, in situ hybridization experiment was performed. According to the Southern blot analysis, a large amount of HBV DNA was present in the cytoplasm when compared with that in the nucleus (Fig. 2). To efficiently trace the nuclear localization of HBV DNA, a plus-strand specific probe was

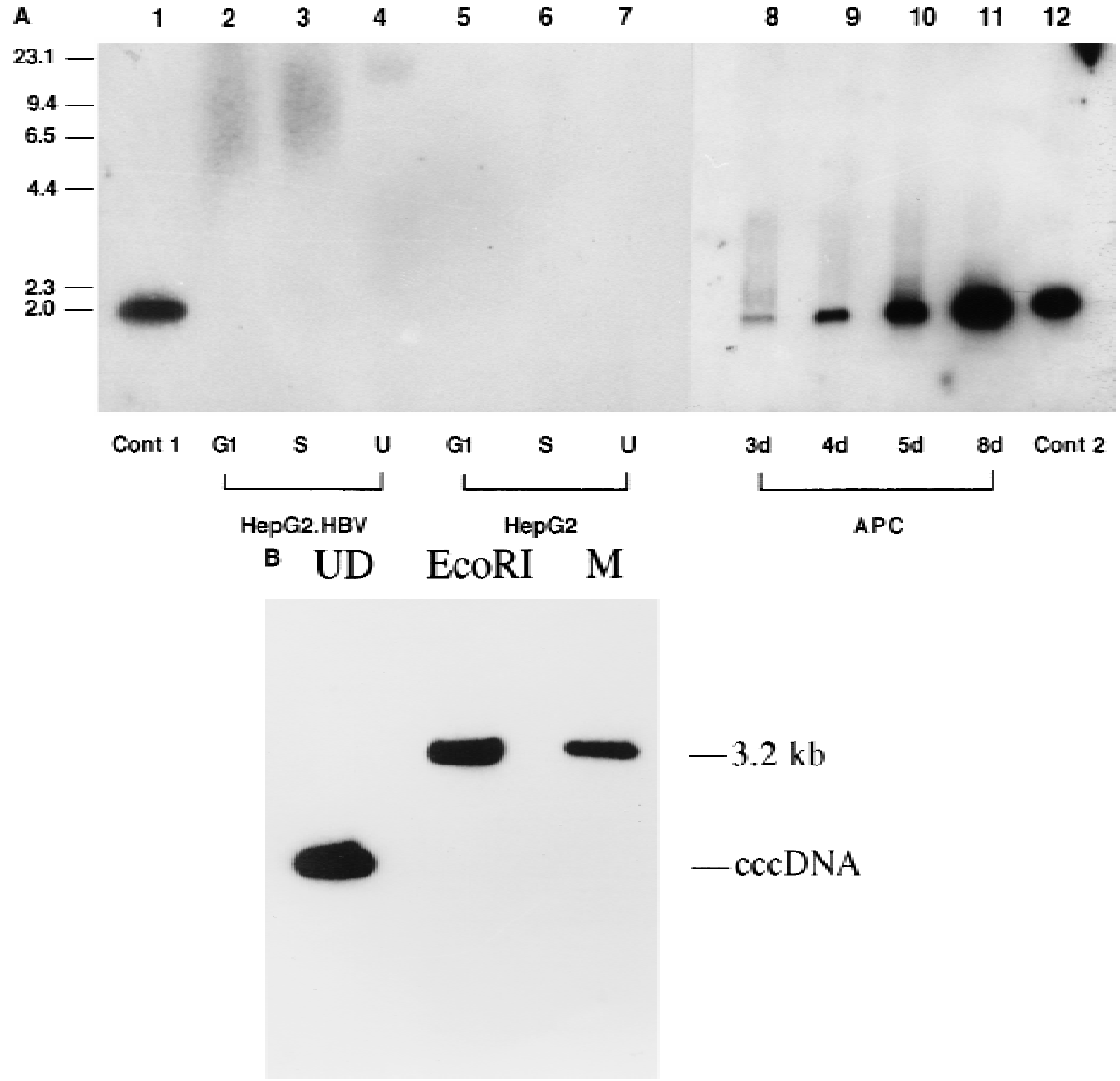

Fig. 4. (A) Detection of cccDNA in HepG2.HBV cells. HepG2 (lanes 5–7) and HepG2.HBV (lanes 2–4) cells were either not synchronized (lanes 4 and 7) or synchronized by serum starvation. Cells were harvested at 6 hr (G_1; lanes 2 and 5) and 13 hr (S; lanes 3 and 6) after serum supplement. HBV cccDNA was extracted by Hirt fractionation without protease K digestion (Materials and Methods). As a control (Cont 1; lane 1), HepG2 cells were mixed with 10 pg of a plasmid, pECE-fC (3.3 kb, containing HBV sequence, see Material and Methods), and the DNA was extracted in parallel with other samples. HepG2.HBV cells were also incubated in 2 μg of aphidicolin (APC; lanes 8–11) for up to 9 days. The cells were harvested at day 1 through 9 after addition of aphidicolin. Only the results of day 3 to 8 were shown here. A 2.0 kb HBV DNA fragment (*EcoR*I-*Xba*I of pECE-C) served as a control (Cont 2; lane 12). B) *EcoR*I digestion of cccDNA. HepG2.HBV cells were incubated in 2 μg of aphidicolin for 4 days. Nuclear cccDNA was extracted by Hirt fractionation. After extraction, DNA was either incubated with only buffer (UD: undigested), or *EcoR*I (EcoRI) for 1 hr. pECE-fC (3.3 kb) was also digested with EcoRI to serve as a control (M).

designed, which detected only plus- and double-strand HBV DNA (RC and cccDNA). Firstly, the plus-strand specific probe was generated by single primer PCR (Fig. 5). This digoxigenin-labeled probe specifically detected only plus-strand (Fig. 5; lanes 1 and 1′) but not minus-strand (lanes 2 and 2′) HBV DNA. Even minor amount of plus-strand DNA which was not seen by ethidium bromide staining (lane 1) can still be detected (background smear in lane 1′). The probe also detected double-stranded HBV DNA (lane 3 and 3′). This probe was then used to perform in situ hybridization experiment (Fig. 6). Cells synchronized by serum-starvation were analyzed at 6 (G_1 phase; Fig. 6A), 10 (early S; Fig. 6C) and 13 hr (S phase; Fig. 6B) after serum supplement. Only parts of the slides representing the major staining patterns were shown here. By counting the cells, HBV DNA was localized predominantly in the nuclei of 81%, 26% and 8% of HepG2 cells at 6 (G1), 10 and 13 hr (S), and predominantly in the cytoplasm in 5%, 45%, and 88% of cells, respectively. For the remaining part of cells, HBV DNA was distributed equally between the cytoplasm and nuclei.

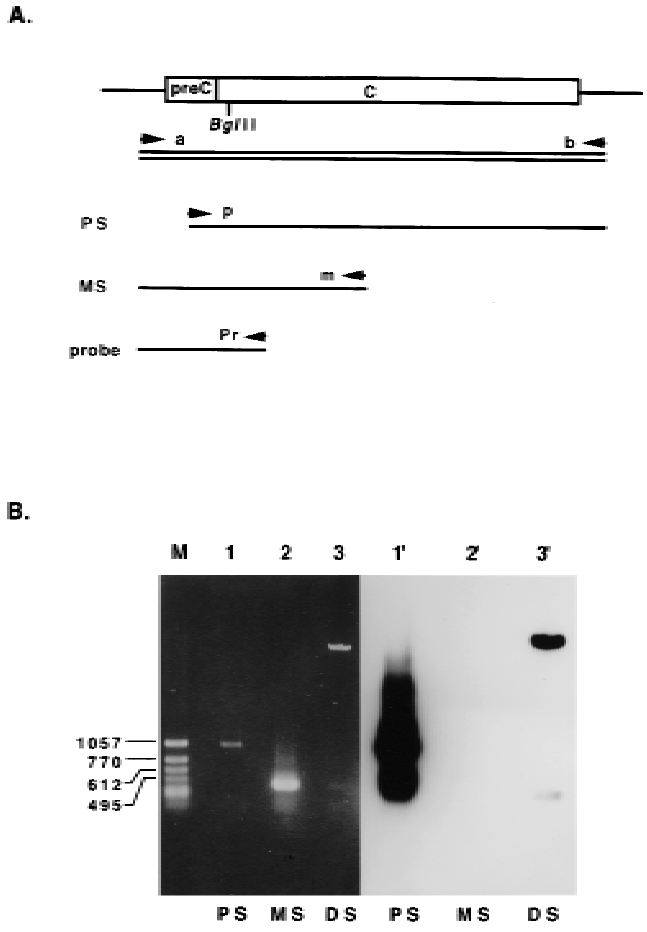

Fig. 5. Detection of HBV DNA using plus-strand specific, digoxigenin-labeled probe. (A) Generation of plus-strand specific probe. The positions and sequences of primers used for generation of probe (Pr), minus-strand control (m), plus-strand control (p), and double-strand control (a and b) were described in Materials and Methods. The probe was labeled with digoxigenin during PCR. The double-strand control was first digested with *Bgl*II before used. (B) Specificity of the digoxigenin-labeled, plus-strand specific probe. Lanes 1 to 3, agarose gel stained with ethidium bromide; lanes 1′ to 3′, southern analysis of the same gel using the specific probe. M: DNA marker (øX/*Hinc*II); PS: plus strand control; MS: minus strand control; DS: double strand control.

DISCUSSION

During the natural course of chronic hepatitis B, three clinical phases can be identified: immune tolerance, immune clearance, and residual stages [Chen, 1993]. Transition from early to late phases is accompanied by decrease of nuclear viral core antigen (HBcAg) with concomitant increase of cytoplasmic HBcAg [Chu et al., 1995]. Previous studies showed that the nuclear transport of core protein is cell cycle related, although different mechanisms maybe involved using different systems. In transfected cell lines as well as human hepatocytes, the core protein localized largely in the nucleus in the $G_{1/0}$ phase but S phase in the cytoplasm [Yeh, 1993; Chu, 1995], whereas in transgenic mice, the core protein accumulated slowly in the nucleus to form particles and was only released to cytoplasm during mitosis [Guidotti, et al., 1994]. Although inconsistency exists, these data carried an important message that the presence of cytoplasmic core protein indicated proliferation (regeneration) of hepatocytes. Since nuclear transport of HBV core protein may be crucial for cccDNA accumulation, one important question would be whether the nuclear import of viral DNA is also affected by cell cycle.

It was found that nuclear localization of HBV DNA (RC form) occurred only in the G_1 phase and prolonged aphidicoline treatment was required for cccDNA formation. A possible explanation of these results is that hepatocytes in different cell cycle phases allowed different direction of viral DNA traffic. In the G_1 phase hepatocytes, HBV DNA is recycled into the nucleus to form cccDNA and only a small amount is destined for virion maturation. This small but constant out-flow of virion will eventually resulted in a high concentration of serum HBV DNA. Furthermore, accumulation of cccDNA results in an increase of total intracellular HBV DNA for viron maturation. In contrast, in the S phase hepatocytes, nuclear entry of HBV DNA dose not occur and a larger amount of the viral DNA was destined for virion maturation. Although the instant outflow of virion is large, degradation of cccDNA after several half-lives eventually led to clearance of HBV DNA [Civitico and Locarnini, 1994]. In this view, a constantly regenerated liver is unfavorable for HBV replication because the G_1 phase may not be long enough for cccDNA accumulation. This explained a recent observation that HBV DNA replication is enhanced in quiescent hepatocytes [Ozer et al., 1996].

The explanation provided above is based on an assumption that the RC form of HBV DNA is being degraded constantly in the nuclei with a relatively short half-life. Therefore, when viral DNA is not transported into the nuclei, no HBV DNA can be detected. However, this may not be the case. An alternative interpretation of the data is that the RC form of nuclear HBV DNA is transported out of the nuclei during the S phase through an unknown mechanism. Also, HBV DNA may not be imported into the nuclei during the G_1 phase, but instead, it diffuses from the cytoplasm into the nuclei during the M phase and remains there. In this study, we cannot distinquish between these possibilities.

Despite a correlation between cell cycle and nuclear localization of HBV DNA, the role of nuclear core protein still cannot be well defined in this study. In HepG2.HBV cells, small amount of core protein was detected in the nucleus in the G_1 phase (Fig. 1), which seemed encouraging because HBV DNA also localized to the nucleus in G_1 phase. However, in un-synchronized HepG2.HBV cells, when no core protein was found in the nucleus, HBV DNA (RC form) was still detected in the nucleus (Fig. 2). Thus, if the core protein is really required to carry the DNA into the nucleus, only a very small amount is sufficient or, as previous studies have suggested, the nuclear transport of core protein is a by-standing event [Guidotti et al., 1994; Kann et al., 1997].

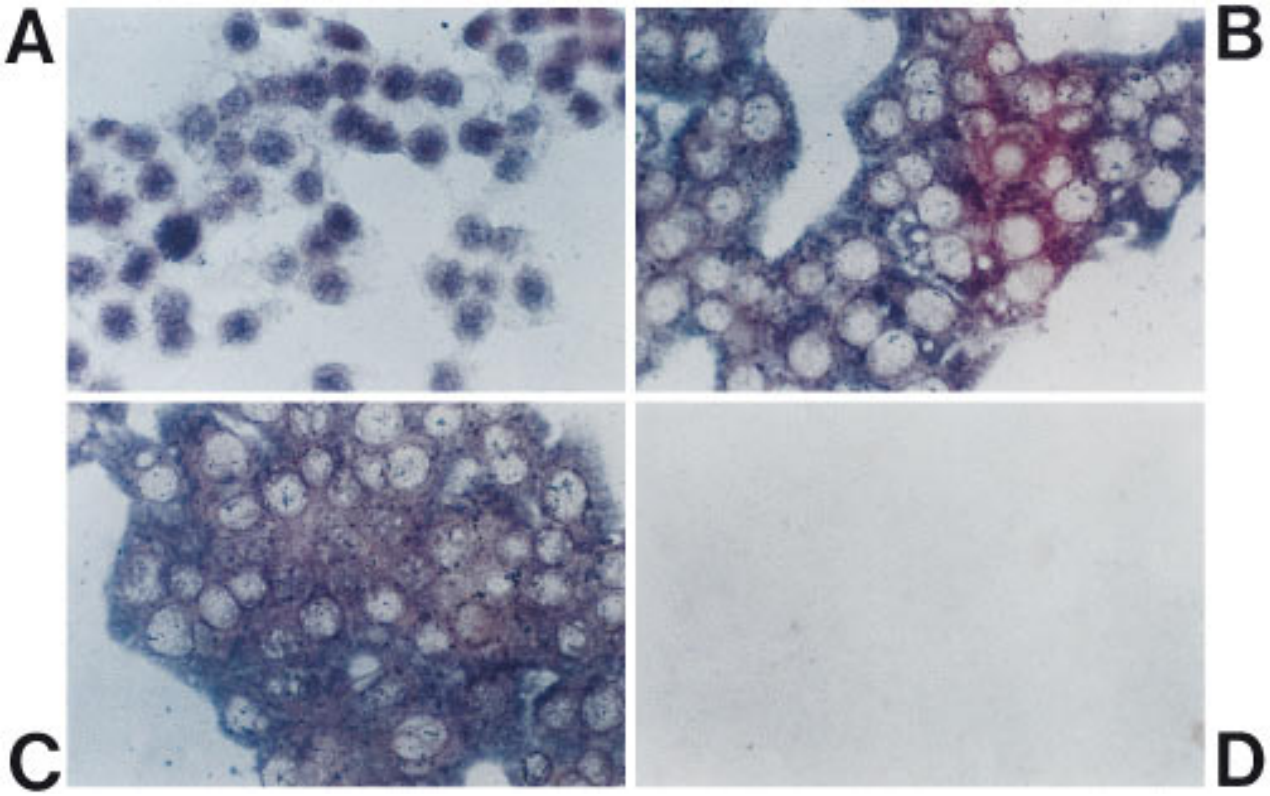

Fig. 6. In situ hybridization using HBV plus-strand specific probe in synchronized HepG2.HBV cells. Cells were grown on cover slip and synchronized with serum starvation. They were fixed at 6 hr (G_1 phase; panel A), 10 hr (G_1/S boundary; panel C), and 13 hr (S phase; panel B) after serum supplement. The original HepG2 cells were used as negative control (panel D). Procedure of in situ hybridization was described in Materials and Methods. Only the representative fields in the slides were shown here. See text for the percentages of cells with nuclear and cytoplasmic viral DNA in each slide.

Subcellular fractionation to analyze the distribution of various forms of HBV DNA has been carried out previously using infected liver cells [Miller and Robinson, 1984]. Our results are consistent with this report except that in the nuclei of primary liver cells, a large amount of cccDNA was detected. In our case, we can only detect cccDNA after prolonged incubation of the cells in aphidicolin. Although other mechanisms such as an unidentified drug effect can also be responsible, the most likely explanation is the result of a protracted G_1 phase. This allowed the nuclear RC viral DNA to be processed (excision of polymerase protein, ligation, DNA repair, etc.) and became cccDNA or, the G_0 phase was required, as in primary liver cells, for cccDNA formation. Of note is that a prolonged G_1 phase is not the only requirement for cccDNA formation. In transgenic mice, no cccDNA can be found in the nuclei, although most hepatocytes should be in the $G_{1/0}$ phase [Guidotti et al., 1994]. Obviously, other cellular factors (species specific) must be involved. Another interesting observation is that the SS viral DNA cannot enter the nucleus. It is likely that the nucleocapsid has to mature to a certain stage in order to recycle its viral DNA. In contrast, the SS viral DNA can be detected in the medium (Fig. 3), though in a very small amount, indicating a looser restriction for the maturity of nucleocapsid to be packaged and secreted.

Although nuclear cccDNA has been clearly demonstrated in the human hepatocyte, only a few studies can detect it in the nucleus by in situ hybridization [Blum et al., 1983; Blum et al, 1984; Wirth et al., 1992; Naomov et al., 1993; Wu et al., 1996]. Our Southern blot analysis revealed a large amount of HBV DNA in the cytoplasm. As a result, the nuclear HBV DNA if in a small amount will be regarded as background. Therefore, we made use of a plus-strand specific probe to detect only the plus-strand HBV DNA. The result of in situ hybridization using this probe is generally consistent with that of southern analysis except that in the S phase, there is still 8% of cells having nuclear HBV DNA by in situ hybridization, which cannot be detected by subcellular fractionation experiment. It is possibly caused by the loss of nuclear viral DNA during the procedure of fractionation.

In summary, it was found that only RC viral DNA is allowed to enter the nucleus and the entry is cell cycle dependent. Most importantly, the formation of cccDNA required a protracted G_1 phase in HepG2.HBV cells.

ACKNOWLEDGMENTS

We thank M.H. Tsai and S.C. Chu for preparing the manuscript.

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